PROLONGED INHIBITION OF MOTOR ACTIVITY FOLLOWING REPEATED EXPOSURE TO LOW LEVELS OF CHEMICAL WARFARE AGENT VX

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Research was supported by government funding. The views of the authors do not purport to reflect the position of the Dept. of the Army or the Dept. of Defense (paragraph 4-3), AR 360-5.

ABSTRACT

While neurobehavioral effects of acute exposure to toxic levels of chemical warfare nerve agents (CWNA) have been characterized (e.g. [8]), much less is known about the effects of repeated exposure to non-convulsive levels of CWNA. In Exp. 1, male mice that received repeated exposure (1/day x 5 days/wk x 2 wk) to 0.4 LD_{50} of the nerve agent VX had much lower activity in the home cage, relative to saline treated mice, with activity levels gradually reaching that of control by 6 weeks post-exposure. In Exp. 2, repeated exposure to 0.2 LD_{50} and 0.4 LD_{50} VX in male and female mice reduced activity in a novel open-field test 10 days following the last VX exposure. These findings indicate long-term performance deficits following exposure to non-convulsive levels of VX.

INTRODUCTION

Chemical warfare nerve agents (CWNA) are highly toxic organophosphorus (OP) chemicals that irreversibly inhibit many serine esterases, including acetylcholine esterase (AChE) in the central and peripheral nervous system. Prolonged inhibition of AChE increases acetylcholine (ACh) at neuronal synapses and at the neuromuscular junction, and can cause acute toxicity. Toxic symptoms include convulsions, tremors, and bronchial constriction, which can then lead to asphyxiation and death [21]. In addition, symptoms of sleep disturbances, psychomotor retardation, and intellectual impairment have been reported following OP exposure. Pathological changes, including neuronal necrosis and axonal degeneration, are present in the hippocampus of rats surviving acute soman, with most behavioral and neuropathological effects of CWNA observed only in animals that display seizures [13,14,20].

While acute effects of CWNA on cognition and behavior are well characterized, much less is known about the effects of repeated exposure to sub-toxic levels of CWNA, including VX (O-ethyl S-[2(diisopropylamino)ethyl]methylphosphonothioate). Most of the available literature on repeated exposure to low levels of organophosphorus compounds (OP) in humans is based on findings of repeated exposure to pesticides, some of which may elicit similar pathology but are typically much less toxic than CWNA. Repeated pesticide exposure has been linked with increased anxiety, depression, and fatigue, as well as impaired psychomotor function [10,16], and subtle cognitive deficits [15]. Incidents such as the release of sarin in the Tokyo subways [18] and the destruction of an ammunition depot containing sarin and cyclosarin potentially exposing civilians and soldiers to low level of CWNA (reviewed in [12]), have

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 OCT 2005	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Prolonged Inhibition Of Motor Activity Following Repeated Exposure To Low Levels Of Chemical Warfare Agent VX	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Division of Neuroscience, Walter Reed Army Institute of Research, Silver Spring, MD 20910-7500 USA**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM001851, Proceedings of the 2003 Joint Service Scientific Conference on Chemical & Biological Defense Research, 17-20 November 2003. , The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**8**	

increased awareness of the need to understand the short-and long-term effects of sub-lethal CWNA exposure.

Acute exposure to toxic levels of soman decreases locomotor and rearing activity in mice [4], and impairs spatial memory in rats [11]. In mice, Baille et al. observed that a sub-convulsive dose of soman decreased locomotion 30 min and 24 hr, but not 7 days after exposure [2]. These authors used the total distance traveled in an elevated plus maze as an index of locomotor and exploratory activity. In rats, acute exposure to sub-convulsive levels of soman and sarin reduces activity (rearing, ambulation) in the open field for at least one hour after exposure [17]. There has been limited research on the neurobehavioral effects of toxic or sub-toxic levels of VX. We hypothesized that repeated exposure to sub-convulsive doses of VX would reduce motor activity in mice.

In the current experiment, we evaluated whether repeated exposure (5 days/wk for 2 wks) to sub-convulsive levels of VX affected activity within the home cage and within a novel environment. We evaluated home cage activity (24 hr/day) in male mice following repeated exposure to 0.4 LD_{50} VX or saline. In addition, we measured locomotor and exploratory activity in male and female mice in the novel open field environment, following exposure to 0.2 LD_{50} VX, 0.4 LD_{50} VX or saline. Our current findings lend support for long-term motor inhibition effects at sub-convulsive exposures to CWNA.

METHODS

OVERVIEW

Principles of laboratory animal care (NIH publication No. 85-23, revised 1985) were followed. Research was conducted under protocols approved at both the WRAIR IACUC and the USAMRICD IACUC. Mice were exposed to VX or saline at USAMRICD over a 2 week period, and were then transported to WRAIR (61 miles from USAMRICD) after their last exposure, for behavioral assessment. In Experiment 1, male mice were placed into home cage activity monitors one week after their last exposure to 0.4 LD_{50} VX or saline, and activity was continuously monitored for the following four months. In Experiment 2, male and female mice were tested for exploratory and locomotor activity in an open field test one week after the last exposure to 0.2 LD_{50} VX, 0.4 LD_{50} VX or saline.

ANIMALS

Mice (25-30 g; Jackson Laboratories) were group-housed (6/cage) upon arrival at USAMRICD under reverse light-dark cycle (lights off at 1100), with food and water available ad libitum. One week before agent exposures, mice were implanted with subcutaneous identification chips (BioMedic Data Systems, Inc., Seaford, Delaware). Following agent exposure, mice were transporte to WRAIR and housed under reverse light-dark cycle (lights off at 0900), with food and water available ad libitum. During transport, gelpacks were provided to prevent dehydration and cage mates were transported in together in each box. At WRAIR, mice in Exp. 1 were individually housed in Plexiglas cages 48 x 27 x 20 cm in size, while in Exp. 2, male and female mice were housed separately (6/cage).

DRUG EXPOSURE

In Exp. 1, male mice received acute subcutaneous (s.c.) injections of either 0.4 LD_{50} VX (8.4 µg/kg; n=6) or saline (n=6) once per day Monday through Friday at 0830 $\pm$ 1 hr for 2 weeks, totaling 10 exposures. In Exp. 2, male and female mice received acute injections (s.c.) of 0.2 LD_{50} VX (4.2 µg/kg; n=13 male, 15 female), 0.4 LD_{50} VX (8.4 µg/kg; n=15 male, 13 female), or saline (n=13 male, 12 female) once per day Monday through Friday at 0830 $\pm$ 1 hr for 2 weeks, totaling 10 exposures. Injection volume was 1 ml/kg. Dilute agent was obtained from USARMICD's surety issue laboratory. Agent was diluted in saline solution (Phoenix Scientific Inc.) and aliquoted into vials for daily injection so that mice received the same dilution and lot over the course of the two weeks.

HOME CAGE ACTIVITY (Experiment 1)

One week after the last exposure, home cage activity was monitored 24 hr/day in 6 mice from each group, using a Cage Rack System (Photobeam Activity System; San Diego Inst., CA). The total number

of photobeam interruptions was used to indicate total activity, while two successive photobeam interruptions within 10 sec indicated ambulatory activity.

OPEN FIELD TEST (Experiment 2)

One week after the last exposure, mice were tested in a novel open field test (47 x 48 cm; Accuscan) for 15 min. A 60-watt light bulb was placed 90 cm above the center of each open field to provide a bright area in the center of the open field, and darker areas along the perimeter. The open field is used to measure both exploratory activity and as a screen for anxiolytics. Anxious mice tend to avoid the brightly lit center of the maze, and display thigmotaxis (wall following), in which they closely walk along the perimeter of the open field. At the start of the test, the mouse was placed in the center of the open field. Sixteen photocells along each edge of the open field recorded the mouse's position and movement.

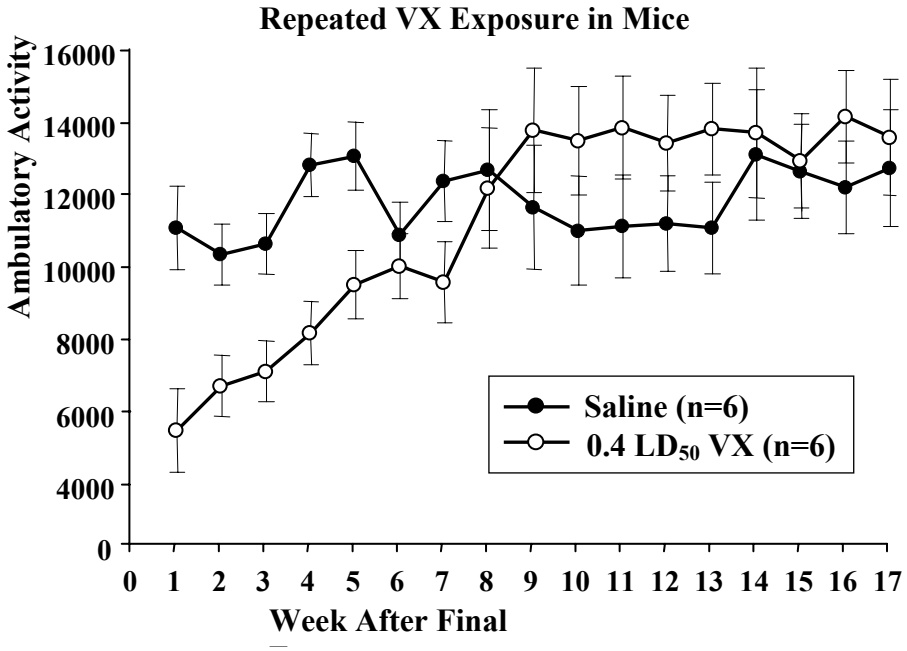
STATISTICS

For the home cage activity data, a repeated measures analysis of variance (ANOVA) was used with drug treatment as a between factor and activity measures (total activity, ambulation) as the repeated measure. Since there was a significant interaction between drug and day of activity measure, a one-way ANOVA was performed for each treatment group, and t-tests were used to compare activity levels between groups on each day. For the open field data, a two-way ANOVA was used, with drug dose and sex as between factors, to evaluate different measures of activity (ambulatory activity, total activity).

RESULTS

Although mice that received 0.4 LD_{50} VX did not have convulsions, these mice did have transient, mild clinical signs, including tremors, fasciculations, and reduced body temperature for several hours following their 5^{th}, 9^{th} and 10^{th} exposures. In the subsequent weeks following the last exposure, there were no obvious clinical signs. However, we observed a significant and robust decrease in both total and ambulatory activity in the home cages of mice chronically exposed to 0.4 LD_{50} VX.

Mice that were chronically exposed to 0.4 LD_{50} VX experienced a deficit in activity relative to saline-treated mice for over 5 following exposure (Figures 1-2). There was a significant interaction between test day and treatment group for ambulatory activity ($F(16, 160) = 5.34$; $p<0.001$) and for total activity ($F(16, 160) = 2.33$; $p<0.01$). For ambulatory activity, levels were significantly lower in VX-exposed mice for 5 weeks following exposure, and tended to be lower 7 weeks following exposure. For total activity, activity levels were significantly lower two weeks following VX exposure and tended to be lower four weeks following exposure. Following a large initial deficit, activity levels gradually increased post-exposure. In VX-treated mice, levels of ambulatory activity ($F(16, 80) = 10.58$; $p<0.001$) and total activity ($16, 80) = 5.29$; $p<0.001$) increased in the first 9 weeks following VX exposure, and then leveled off. In saline treated mice, there was no significant change in activity levels over the course of the study. As expected, mice were more active during their dark cycle than their light cycle, with VX inhibition of activity occurred during both the light and dark cycle. Inhibition of activity was more apparent during the dark cycle. (Figure 3).



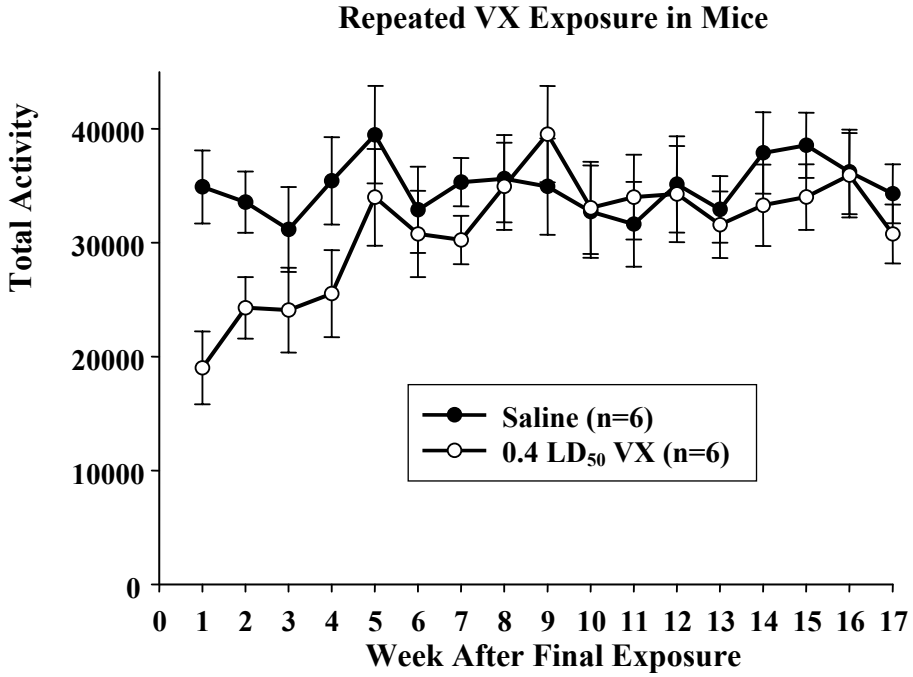


Figure 2. C57BL/6 mice that received repeated exposure to 0.4 LD_{50} VX (1/day x 5 days/week x 2 weeks) displayed less total activity, relative to vehicle-treated mice. Activity returned to baseline by 3 weeks after exposure.

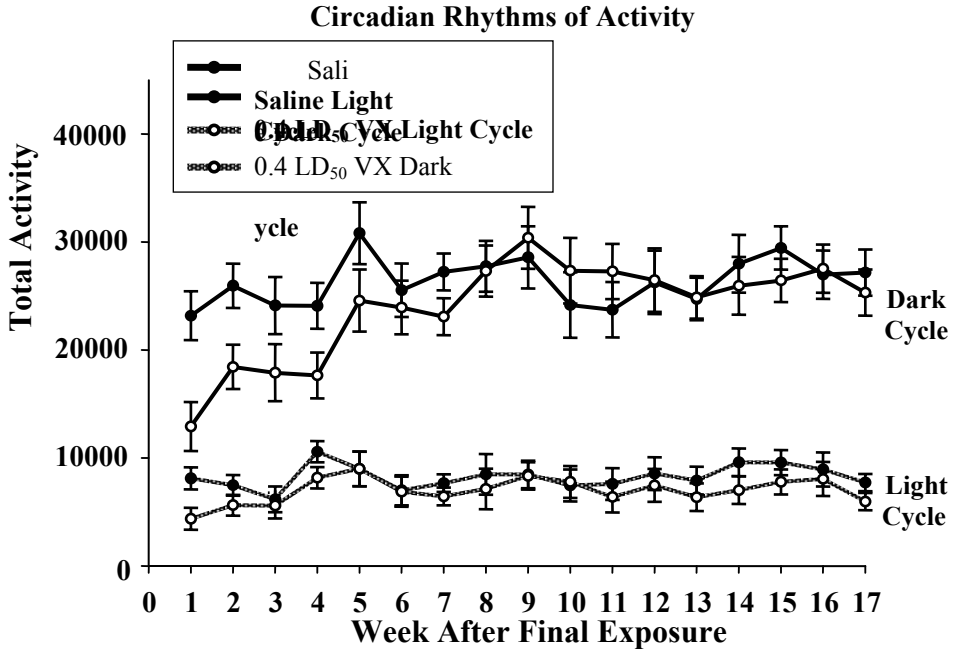


Figure 3. C57BL/6 mice repeatedly exposed to 0.4 LD_{50} VX 1/day x 5 days/week x 2 weeks) displayed less total activity during both the dark and light cycles, relative to saline-treated mice. Activity levels in the dark cycle reached control levels by 6 weeks post-VX and that in the light cycle reached control by 3 weeks post-VX.

A similar immobilizing effect of VX was observed in the open field in both male and female mice 1 week after the last VX exposure. There was a main effect of VX on activity, with VX significantly reducing total horizontal activity (sum of all non-rearing movements) in VX-treated mice ($F(2, 67) = 7.09$; $p<0.01$; Figure 4). Horizontal activity was reduced by both 0.2 LD_{50} VX and 0.4 LD_{50} VX, relative to control mice.

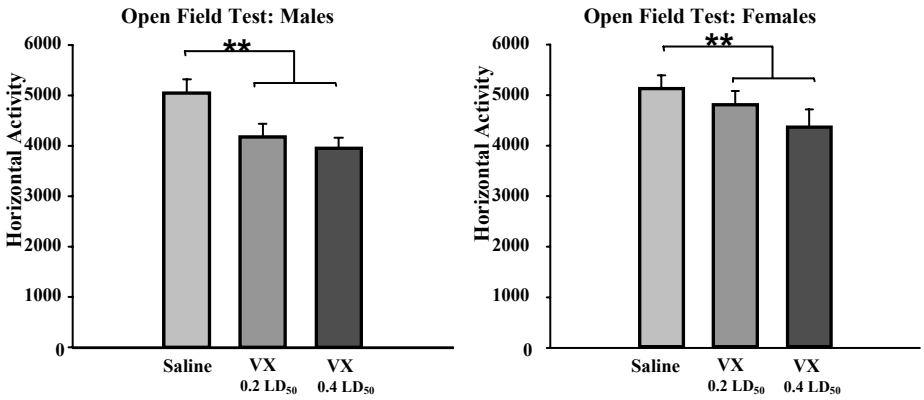


Figure 4. Mice were placed in a novel open field environment for 15 min. Photocells embedded around the perimeter of the open field measure the animal's locomotor and rearing behavior. Male (A) and female (B) mice

There were sex differences in activity levels. Female mice had more total distance ($F(1,67) = 6.9$; $p=0.01$), and a greater duration of movement ($F(1,67) = 4.7$; $p<0.04$), while male mice had more rearing behavior ($F(1, 67) = 14.3$; $p<0.001$). In addition, female mice had greater thigmotaxis ($F(1, 67) = 10.9$; $p=0.002$), relative to male mice. Thigmotaxis or wall-following is considered a measure of anxiety since it is reversed by anxiolytics (e.g. [5]), and consists of closely walking along the perimeter of the open field, in the darker areas of the open field.

There was no significant difference between groups in body weight throughout the course of the study. With time, all mice had significantly increased body weight, regardless of treatment group.

DISCUSSION

We observed that mice exposed repeatedly to 0.4 LD_{50} VX had dramatically less total activity and ambulatory activity than control mice in the weeks after repeated exposure to sub-convulsive levels of VX. This large inhibition of motor activity was prolonged, gradually returning to that of control by the 6th week following the last VX exposure. There was variability in individual motor response to repeated exposure to VX, with some mice more affected than others. In guinea pigs, Dr. Petras (personal communication) observed neuropathology in some, but not all guinea pigs repeatedly exposed to $0.4LD_{50}$ sarin. These current findings of prolonged inhibition activity need to be replicated with larger sample sizes and additional doses should be evaluated. In addition, identifying the time point at which hypoactivity first occurs is important to determine, since this may have implications for human performance if exposed for prolonged periods of time to analogous dose levels.

We also observed that male and female mice had less activity in a novel environment (open field test), relative to control, when tested 10 days following the last VX exposure. The inhibition of activity by VX in the 15 min open field test was not as large the inhibition we observed in the home cage. We also observed that female mice had more thigmotaxis than male mice in the open field test, but that this parameter was not affected by VX. Thigmotaxis is considered a measure of anxiety.

We previously observed that body temperature was reduced following the last exposure to a sub-convulsive dose of VX (unpublished data), which may be linked to the reduced motor activity. However, the reduced activity continued for much longer than the reduced body temperature, the latter of which returns to normal by 24 hr following the last exposure. Clark et al. (2002) observed inhibition of the acoustic startle response (ASR) in 129/Sv mice exposed repeatedly to $0.4LD_{50}$ VX [6]. However, the effects of VX on startle were transient, only occurring shortly after the exposures. Transient effects, such as inhibition of ASR and reduction in body temperature may result from cumulative toxicity. For both of these studies, $0.4LD_{50}$ VX was administered daily and clinical signs appeared following the 5^{th} exposure and again following the 9^{th} and 10^{th} exposure. Since mice were not exposed on the weekend, there would be less cumulative toxicity after the 6^{th} exposure than following the 5^{th} exposure.

Acute exposure of rats to sub-toxic levels of soman reduced motor activity in the open field for at least 1 hr after exposure [17]. In mice, activity levels were reduced 24 hr, but not 7 days, following acute exposure to sub-toxic levels of soman [2]. The doses tested led to ataxia and tremors, but rarely convulsions. Clinical signs of tremors were gone by 24 hr after exposure, but hypoactivity continued. In the study by Baille et al., activity in mice was assessed by total distance traveled in the elevated plus maze, as an index of locomotor and exploratory activity. Our current findings suggest that repeated exposures to OP's may lead to longer lasting motor inhibition.

Much less is known about neurobehavioral effects of repeated exposure to organophosphorus compounds. Ali et al. observed that repeated exposure (10 days) to dichlorvos (i.p.) reduced motor activity in the open field [1]. Both rearing and ambulatory activity were reduced, with partial recovery by the 10 day of exposure. Time periods of recovery following exposure were not discussed. However, Socko et al. did not observe changes in performance in the open field following repeated exposure to dichlorvos [22]. In our study, inhibition of motor activity following repeated exposure to the more toxic chemical VX continued for weeks after exposure. Note, that in our study, mice were tested only one time in the open field, to use this test as a novel environment. In the experiments by both Ali et al. and Socko

et al., animals were tested in the open field multiple times, which can affect their performance, reducing activity as animals habituate to the test situation, and the test loses its "novelty".

It is unclear whether the effects of repeated VX exposure on motor activity are mediated centrally or by effects on the neuromuscular junction. In macaques, acute exposure to soman resulted in skeletal muscle changes 10 days following the exposure [3]. These skeletal changes were mild but occurred regardless of whether the macaques displayed convulsions. In rats, soman induced necrosis in a small number of myofibers in muscle bundles that had fasciculations, but these effects only lasted 7 days [9]. In another study in rats, ultrastructural damage was revealed at and near the motor endplates and was observed for up to 3 weeks after acute soman exposure [9,19]. Much less research has been done on the neurobehavioral effects of repeated OP exposure. There is, however, some evidence that long term neuromuscular effects may continue for weeks after a chronic OP exposure, as measured by EPP jitter [7].

CONCLUSION

The current findings of prolonged hypoactivity following repeated exposure to sub-convulsive levels of nerve agent VX have important implications for potential operational performance deficits following repeated CWNA exposure in humans.

References:

[1] S.F. Ali, O. Chandra and M. Hasan, Effects of an organophosphate (dichlorvos) on open field behavior and locomotor activity: correlation with regional brain monoamine levels, Psychopharmacology (Berl), 68 (1980) 37-42.

[2] V. Baille, F. Dorandeu, P. Carpentier, J.C. Bizot, P. Filliat, E. Four, J. Denis and G. Lallement, Acute exposure to a low or mild dose of soman: biochemical, behavioral and histopathological effects, Pharmacol Biochem Behav, 69 (2001) 561-569.

[3] J.O. Britt, Jr., J.L. Martin, C.V. Okerberg and E.J. Dick, Jr., Histopathologic changes in the brain, heart, and skeletal muscle of rhesus macaques, ten days after exposure to soman (an organophosphorus nerve agent), Comp Med, 50 (2000) 133-139.

[4] J.J. Buccafusco, J.H. Graham and R.S. Aronstam, Behavioral effects of toxic doses of soman, an organophosphate cholinesterase inhibitor, in the rat: protection afforded by clonidine, Pharmacol Biochem Behav, 29 (1988) 309-313.

[5] E. Choleris, A.W. Thomas, M. Kavaliers and F.S. Prato, A detailed ethological analysis of the mouse open field test: effects of diazepam, chlordiazepoxide and an extremely low frequency pulsed magnetic field, Neurosci Biobehav Rev, 25 (2001) 235-260.

[6] M.G. Clark, M.L. Sipos, H. Lukefahr, S.V. Burchnell, E. Sistrunk and E.G. Midboe, Effects of subacute exposure to VX on the acoustic startle response in 129 SVPasIcoCrlBR mice, Bioscience Review Abstracts, (abstract) (2002) 119.

[7] G.E. de Blaquiere, L. Waters, P.G. Blain and F.M. Williams, Electrophysiological and biochemical effects of single and multiple doses of the organophosphate diazinon in the mouse, Toxicol Appl Pharmacol, 166 (2000) 81-91.

[8] D.M. de Groot, E.P. Bierman, P.L. Bruijnzeel, P. Carpentier, B.M. Kulig, G. Lallement, B.P. Melchers, I.H. Philippens and A.H. van Huygevoort, Beneficial effects of TCP on soman intoxication in guinea pigs: seizures, brain damage and learning behaviour, J Appl Toxicol, 21 Suppl 1 (2001) S57-65.

[9] W.D. Dettbarn, Pesticide induced muscle necrosis: mechanisms and prevention, Fundam Appl Toxicol, 4 (1984) S18-26.

[10] N. Fiedler, H. Kipen, K. Kelly-McNeil and R. Fenske, Long-term use of organophosphates and neuropsychological performance, Am J Ind Med, 32 (1997) 487-496.
[11] P. Filliat, D. Baubichon, M.F. Burckhart, I. Pernot-Marino, A. Foquin, C. Masqueliez, C. Perrichon, P. Carpentier and G. Lallement, Memory impairment after soman intoxication in rat: correlation with central neuropathology. Improvement with anticholinergic and antiglutamatergic therapeutics, Neurotoxicology, 20 (1999) 535-549.
[12] L.A. McCauley, M. Lasarev, D. Sticker, D.G. Rischitelli and P.S. Spencer, Illness experience of Gulf War veterans possibly exposed to chemical warfare agents, Am J Prev Med, 23 (2002) 200-206.
[13] J.H. McDonough, Jr., T.R. Clark, T.W. Slone, Jr., D. Zoeffel, K. Brown, S. Kim and C.D. Smith, Neural lesions in the rat and their relationship to EEG delta activity following seizures induced by the nerve agent soman, Neurotoxicology, 19 (1998) 381-391.
[14] J.H. McDonough, Jr., L.W. Dochterman, C.D. Smith and T.M. Shih, Protection against nerve agent-induced neuropathology, but not cardiac pathology, is associated with the anticonvulsant action of drug treatment, Neurotoxicology, 16 (1995) 123-132.
[15] U.K. Misra, M. Prasad and C.M. Pandey, A study of cognitive functions and event related potentials following organophosphate exposure, Electromyogr Clin Neurophysiol, 34 (1994) 197-203.
[16] N. Munro, Toxicity of the Organophosphate Chemical Warfare Agents GA, GB, and VX: Implications for Public Protection, Environ Health Perspect, 102 (1994) 18-37.
[17] S.A. Nieminen, A. Lecklin, O. Heikkinen and P. Ylitalo, Acute behavioural effects of the organophosphates sarin and soman in rats, Pharmacol Toxicol, 67 (1990) 36-40.
[18] S. Ohbu, A. Yamashina, N. Takasu, T. Yamaguchi, T. Murai, K. Nakano, Y. Matsui, R. Mikami, K. Sakurai and S. Hinohara, Sarin poisoning on Tokyo subway, South Med J, 90 (1997) 587-593.
[19] J.P. Petrali, K.R. Mills, D.M. Maxwell and M.D. Green, Soman-induced myopathy, Anatomical Records, 208 (1984) 474.
[20] J.M. Petras, Soman neurotoxicity, Fundam Appl Toxicol, 1 (1981) 242.
[21] R.W. Russell and D.H. Overstreet, Mechanisms underlying sensitivity to organophosphorus anticholinesterase compounds, Prog Neurobiol, 28 (1987) 97-129.
[22] R. Socko, S. Gralewicz and R. Gorny, Long-term behavioural effects of a repeated exposure to chlorphenvinphos in rats, Int J Occup Med Environ Health, 12 (1999) 105-117.